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POTENCY OF ANTIMENINGOCOCCIC AND ANTIPNEUMOCOCCIC SERUMS.

Since December, 1917, all lots of serum intended for use against meningococcus infection or pneumococcus infection (Type I), and subject to control by the Treasury Department, have been tested for potency at the Hygienic Laboratory. Tests for potency are made at the manufacturing establishments, but before being released for interstate sale, each lot must pass the official test at the Hygienic Laboratory. Under these provisions, 461 lots of antimeningococcic serum and 781 lots of antipneumococcic serum have been tested. Numerous tests have also been made on serums from other sources.

In addition to the usual tests for sterility, the following are the minimum requirements:

Duplicate samples of 15cc. each are to be submitted of each lot as ready for the final container, after all treatments, such as the mixing in of preservatives and filtration, are completed, and after the lot has passed satisfactory tests at the manufacturing establishment. Protocols of these tests shall be sent to the Hygienic Laboratory.

The serums are to be free from excessive color or turbidity. The maximum intensity of color is that represented by a 0.3 per cent saturated solution of bismarck brown in absolute alcohol, and the maximum turbidity that of 200 parts per million of silica. There should be no gross particles in the serum.

Antimeningococcic serums are considered satisfactory which show an agglutination titer or complement fixation titer against each antigen at least equal to that shown by the control serum furnished by the Hygienic Laboratory.

Antipneumococcic serums are considered satisfactory which show protection of at least as great a percentage of mice as are protected by the control serum furnished by the Hygienic Laboratory. At least two tests of three mice each shall be made of each serum, not less than 24 hours apart, and the total resultant protection shall be compared with the protection of the two sets of mice receiving the control serum. No set of tests shall be considered except those which show some mortality, but not complete, with the control serum.

Method of Performing Agglutination Test for Antimeningococcic Serum.

The antigen.—Strains of meningococci which have been classified by absorption of agglutinin are selected, and are identified in accordance with the following criteria: Gram-negative diplococci which ferment glucose and maltose, but not saccharose, in 24 hours, at 37°; which agglutinate, respectively, with each of four specific rabbit serums, and by preference show no cross-agglutination in a dilution of 1:50 of a serum which will agglutinate homologous cocci in a dilution of 1:400. The strains for antigen production are at present identified as follows:

For Antigen I, H. L. No. 123, originally Rockefeller Institute No. 85.
For Antigen II, H. L. No. 55, originally Rockefeller Institute No. 1.
For Antigen III, H. L. No. 57, originally Rockefeller Institute No. 30.
For Antigen IV, H. L. No. 60, originally Rockefeller Institute No. 81.

Preliminary to preparing an antigen, the strain should be transplanted daily on glucose agar for at least five transfers.

The organism is grown on glucose agar, in Blake bottles, from 12 to 16 hours, at 37° C. The seeding of the glucose agar is made by suspending the 12 to 16 hour growth on a glucose agar slant in 2 cc. of glucose broth, and carrying it over to the Blake bottle with a pipette. The glucose agar used in growing the antigens is of a beef infusion base, 1.5 to 2 per cent agar, 0.5 to 1 per cent glucose, and made to a P_h of 7.5, or 0.3 to 0.5 per cent acid to phenolphthalein by the Fuller scale (hot titration). The agar should, preferably, be cleared without filtration through cotton or paper.

The harvest from the bottles is made by suspending the growth in physiological saline solution, using 10 cc. of saline to suspend the growth of each flask. This suspension is immediately heated at 65° C. for one hour. A purity check by smears stained by Gram's method is made while the stock is heating. After heating, it is diluted with saline (to which phenol is added to make a 0.5 per cent solution) to a turbidity equivalent to that of a silica standard of 1,000 parts per million. To prepare this silica standard, use Pear's precipitated fuller's earth, sift through a 200-mesh sieve, and suspend 1 gram in 1 liter of distilled water, which gives 1,000 parts per million. This turbidity will correspond to approximately 2,000,000,000 meningococci per cc. The turbidity is read in a nephelometer, or can be satisfactorily compared in half-ounce vials (18 by 60 mm.) of uniform thickness. Starting with a small known amount of the suspension to be standardized, the filtered phenolized saline solution is added to bring the suspension to the turbidity of the standard, and the proportional amount then added to the stock.

This diluted antigen can be preserved in the cold room (15° C.) for several weeks with but little autolysis or disintegration.

Antigens giving more than + agglutination with 1:50 dilution of normal horse serum, or less than an average + + + agglutination with 1:100 dilution of the control antimeningococcic serum, should not be used.

The serum.—The dilutions of the serums are made in test tubes of 28 by 150 mm. to permit of thoroughly mixing by blowing dilution and air through a pipette carried to the bottom of the mixture. The lowest dilution tested is 1:50, made by adding 1 cc. of serum to 24 cc. saline, dilution A, and using 0.5 cc. of dilution A and 0.5 cc. of antigen suspension per tube. All dilutions up to 1:400 are made from the first dilution. The dilutions contain 0.02 cc., 0.01 cc., 0.005 cc., 0.0025 cc., 0.00125 cc. (and 0.000625 cc. if necessary) of the serum in 0.5 cc. Dilutions are to be carried high enough to show the extreme titer of the control serum, each dilution being half the strength of the next lower. Intermediate dilutions may be advisable for closer comparison with the control serum.

The test.—All tests are run in parallel series with the control serum, with the two lowest dilutions of normal horse serum and with saline.

The tubes used for the tests are 8 by 75 mm. in size and are set up in metal racks.

The antigen is pipetted into the tubes in 0.5 cc. quantities, followed by the various serum dilutions in equal amount. One pipette is used for delivering all the dilutions of one serum, starting with the most dilute and proceeding toward the lowest dilution. The antigen and serum are mixed by shaking and are incubated for from 16 to 18 hours in a water bath at 56° C.

Readings are made in four degrees of agglutination (indicated by + + + +, + + +, + +, and +, or 4, 3, 2, and 1) and 0 or no agglutination. The degree of clarity of the supernatant fluid after the overnight sedimentation at 56° is taken as the basis for reading these degrees of reaction, and for each day's tests a set of standard tubes is made up for each of the four antigens to serve as criteria for making the readings for that day. To the cloudy supernatant suspension from tubes showing no agglutination (saline or normal serum control tubes) different proportions of saline solution are added to give different degrees of turbidity. Tube A contains 1 cc. of clear saline; tube B (one for each antigen) contains 0.25 cc. of the unagglutinated suspension and 0.75 cc. saline; tube C (one for each antigen) contains 0.5 cc. of the unagglutinated suspension and 0.5 cc. saline; tube D (one for each antigen) contains 0.75 cc. of the unagglutinated suspension and 0.25 cc. saline; tube E (one for each antigen) contains 1 cc. of the unagglutinated suspension; that is, the straight antigen diluted with equal parts of saline or diluted normal serum. A + + + + reaction is complete agglutination, corresponding to tube A—that is, complete sedimentation with water-clear supernatant fluid. A + + + is any degree of agglutination less than this, but giving a turbidity of the supernatant fluid not greater than in tube B of the corresponding antigen. A + + is any degree of agglutination definitely less than + + +, but with a turbidity of the supernatant not greater than in tube C. A + includes agglutinations giving a turbidity of the supernatant fluid definitely greater than that in tube C, but not greater than in tube D. No agglutination, indicated by 0, includes all lower degrees of agglutination corresponding to tube E.

To be satisfactory in agglutinin content a serum should give a + + + reaction with each of the four antigens in dilutions as high as the control serum.

Method of Performing Complement Fixation Test for Antimeningococcic Serum.

I. *The cells.*—The cells used in the test and titrations are the red blood cells of the sheep, washed free of coagulable protein, made up with the amboceptor solution to twenty times the volume of defibrinated blood from which the cells were obtained. After the final washing of the cells they are centrifuged and resuspended in saline solution only up to the original volume of defibrinated blood from which they were obtained; the amount of the final suspension of this strength to be used is therefore 5 per cent of the volume of dilute cell suspension required for the day's tests. The blood of sheep whose cells give irregular or bizarre results in the titration should not be used. The cells are sensitized as follows: The saline needed to make up the required volume—that is, nineteen times as much saline as concentrated cell suspension—is divided into two approximately equal portions, half being added to the necessary amount of amboceptor and half to the concentrated suspension. These two dilutions are then rapidly mixed. The unit of cells is contained in 1 cc. of this mixture. One unit of cells is used in the tests.

II. *The amboceptor.*—The amboceptor used is the serum of a rabbit which has been immunized to red blood cells of the sheep. The undiluted rabbit serum is preserved with 0.25 per cent phenol and kept at 5° C. A unit of amboceptor is that amount which gives complete hemolysis of the unit volume of sheep cells with 0.05 cc. of freshly drawn pooled guinea pig complement; the amounts of amboceptor used in this titration are 0.0001 cc., 0.00015 cc., 0.0002 cc., 0.0003 cc., 0.0005 cc., 0.0007 cc., 0.001 cc., 0.0015 cc., 0.002 cc. Amboceptor should be retitrated every two months.

The amboceptor is diluted for use when mixed with cells as above described (Section I) so that 200 units are contained in each 100 cc. of cell-amboceptor dilution. Two units of amboceptor are used in the tests.

III. *The complement.*—The complement used is that of guinea pig serum with the addition of one-tenth of its volume of saturated (30 per cent) sodium chloride solution, and it is kept below 10° C. This is diluted with three parts of distilled water before use, to make 0.9 per cent NaCl solution. The serum of several pigs bled at one time is pooled for uniformity. The unit of complement is that amount of guinea-pig serum which gives complete hemolysis with two units of amboceptor and one unit of the sheep-cell suspension. In titrating, a mixed antigen containing one-fourth of a unit of each of the four antigens (the resulting mixture corresponding to one full unit) and 1/250 cc. normal horse serum are incubated with each amount of complement for one hour, when the amboceptor and cells are added and the titration read after one hour in the 37° water bath. The amounts of complement used in this titration are 0.02 cc., 0.024 cc., 0.03 cc., 0.036 cc., 0.042 cc., 0.05 cc., 0.06 cc. The complement should have a unitage less than 0.05 cc. The complement is titrated before each test. One unit of complement is used in the tests.

IV. *The antigen.*—The four antigens are made in the same manner as for the agglutination test. The unit of antigen selected is the amount next larger than that which gives a + + + fixation with 1/500 cc. of the control antimeningococcic serum; antigens which, in amounts next larger than the unitage chosen, give any fixation with 1/250 cc. normal horse serum should not be used. The amounts of antigen used in titrating the unit value are 0.01 cc., 0.013 cc., 0.016 cc., 0.02 cc., 0.025 cc., 0.03 cc., 0.04 cc., 0.05 cc., 0.06 cc., 0.08 cc., 0.1 cc., 0.13 cc. Antigens should be retitrated every two months. One unit of antigen is used in the tests.

V. *The serum.*—The serum is not inactivated as a routine, but freshly drawn sera should be inactivated without preservative at 56° for 30 minutes. The dilution of the serum is made in tubes 28 by 150 mm. in size to permit thorough mixing by blowing dilution and air through a pipette carried to the bottom of the tube. The lowest amount tested is 1/250 cc., made by adding 0.4 cc. of serum to 19.6 cc. saline (dilution A) and making dilution B by adding 2 cc. of A to 8 cc. of saline. One cc. of this dilution B contains 1/250 cc. serum. All further dilutions are made from dilutions A or B, to obtain in each cubic centimeter 1/500 cc., 1/1000 cc., 1/2000 cc., and 1/4000 cc. of serum. The dilutions should be carried high enough to show the extreme titer of the control serum.

VI. *The test.*—All tests are run in parallel series with the control serum and with a normal horse serum to which preservative has been added. Each serum is tested with each of the four antigens. The tubes used for the tests are 15 by 120 mm. in size and are set up in metal racks. The final volume used in each tube is 4 cc., allowing 1 cc. each for antigen, serum, and complement, the amboceptor and cells being combined in 1 cc. volume for each tube. The volume in the control tubes is made up to 4 cc. with saline.

A control is used on each serum to determine its anticomplementary value by adding to one tube without antigen the amount of serum used in the lowest test dilution. A control of each antigen is made by setting up a tube with the unit of antigen, without serum. A control is made on the hemolytic system without antigen or serum. A control is made on the sheep cells and amboceptor, without complement, antigen, or serum, to determine the presence of any hemolysis.

Incubation is carried out in a 37° water bath. The antigen-serum-complement mixture is incubated for an hour; then the amboceptor and cells are added and incubation is carried on for a second hour. Readings are made after standing overnight in the cold room at 15°.

Four degrees of positive fixation (+ + + +, + + +, + +, and +, or 4, 3, 2, and 1), and 0 fixation or complete hemolysis are recognized. For each day's tests a set of color standards is made up to serve as a criterion for making the readings for that day. These color standards are made up from completely hemolyzed tubes without visible sediment of red cells, mixed with saline solution in varying proportions to give colors representative of 0 per cent, 25 per cent, 50 per cent, 75 per cent, and 100 per cent hemolysis. Tube A is a tube containing 4 cc. of clear saline, without color (0 hemolysis). Tube B contains 1 cc. from a completely hemolyzed tube and 3 cc. of saline (corresponding to 25 per cent hemolysis). Tube C contains 2 cc. from a completely hemolyzed tube and 2 cc. of saline (corresponding to 50 per cent hemolysis). Tube D contains 3 cc. from a completely hemolyzed tube and 1 cc. of saline (corresponding to 75 per cent hemolysis). Tube E is a completely hemolyzed tube, without added saline.

+ + + +, or complete fixation, should give no color in the supernatant fluid after standing overnight, corresponding to tube A; + + + fixation is taken as any amount of fixation, giving some color in the supernatant fluid, but no deeper than that of tube B; + + fixation corresponds to any degree of hemolysis definitely greater than this but showing no more color than tube C; + includes all slighter degrees of fixation, with an appreciable sediment of red cells; and 0 fixation or complete hemolysis (tube E) should be entirely clear, without sediment.

The titer of a serum is regarded as that dilution at which it gives + + + fixation.

Method of Performing Protection Test for Antipneumococcic Serum.

I. *The culture.*—The culture of Type I pneumococcus should be of such virulence that it is fatal to mice, as a rule, in 0.00,000,001 cc. of an 18-hour beef infusion broth culture. Simultaneously with each day's tests, eight mice are inoculated with the culture alone, receiving no serum, in order to check the virulence of the culture used. These cultural check mice receive 0.00,000,1 cc., 0.00,000,01 cc., 0.00,000,001 cc., and 0.00,000,000,1 cc. of the culture, two mice on each dose. These dilutions are made by adding 1 cc. of the next stronger dilution to 9 cc. broth in tubes 28 mm. in diameter to permit thorough mixing by blowing mixture and air through the pipette which is carried to the bottom of the tube. A separate pipette is used for each dilution. The culture should be checked for purity by plating on blood agar plates previous to each test. At the Hygienic Laboratory the broth for growing the culture is made from beef infused in the ice box, and 2 per cent peptone is used. The culture is passed through mice weekly, 0.5 cc. of an 18-hour broth culture being inoculated intraperitoneally into each of three mice, which are etherized in 5 or 6 hours; about 0.5 cc. of heart's blood is obtained from each mouse and distributed into two tubes of broth and a blood agar plate (for purity test). These tubes are incubated overnight. Three of the six tubes which show a good growth of pneumococcus in pure culture, are selected and stored at 15° as stock cultures. After about 3 days (the day before the test) 0.5 to 1.0 cc. are transplanted from each stock culture to fresh tubes of broth, and plates are made for purity. The stock cultures are quickly put back at 51°, to be used for planting the tubes 18 hours before the next passage through mice. The transplants to be used in the test are incubated for from 16 to 18 hours, and of the 3, those showing a good growth of pure pneumococcus are pooled and diluted for the test. All dilutions of culture are made in broth and should not stand diluted for more than an hour at room temperature.

One-tenth of a cubic centimeter of the culture, diluted so that the dose is contained in 0.5 cc. volume, is given to each mouse, except the cultural check mice.

II. *The serum.*—Two-tenths of a cubic centimeter of each serum (including the control serum), diluted with saline so that this dose is contained in 0.5 cc. volume, is given to each mouse, except the cultural check mice.

III. *The test.*—For each serum tested, at least 3 mice are injected intraperitoneally with 0.2 cc. of the serum, followed within less than 5 minutes by 0.1 cc. of culture, intraperitoneally. Six mice should receive the control serum, 3 at the beginning and 3 at the end of each test. The mice used should weigh 18–22 gms. If there is much variation in weight, they should be arranged in three weight-groups, and a light, a medium, and a heavy mouse chosen for each serum. The mice are observed for 96 hours. All mice counted as fatalities must be proved, by smear and cultures from the heart's blood, to have died with an uncomplicated pneumococcus septicemia. Survivals of less than 12 hours should be regarded as "contaminations." Mice dying of infections, accidents, or from causes other than pure pneumococcus septicemia, as revealed by necropsy, are regarded as "contaminations." Contaminations should be regarded as neither deaths nor survivals, but should be omitted in reading the tests as though they had not been injected. Tests are read 120 hours after injection, to allow for the obtaining of results of necropsies on mice dying on the fourth day. The control serum should protect at least 50 per cent of the mice, on the average. The following data should be included in the protocols of tests:

Date of test, including month, day, and hour.

Operator's initials.

Period of exposure of diluted culture to room temperature.

Period of time necessary for injection of all mice.

Hours of survival of mice dying up to 96 hours, indicated by number.

Survival of mice for 96 hours, indicated by S.

Contamination to be indicated by enclosing in parentheses the number of hours of survival.

PROTOCOL OF TESTS.

(Sample.)

Three mice are injected for each serum used. The serum dose of 0.2 cc. is followed by 0.1 cc. of culture.
"S" indicates survival of 96 hours; contamination is indicated by parentheses.
Cultures passed through mice October 12, 1919.
Dilution of culture began 11.48 a. m.
Injection of serum began 12.02 p. m.
Injection of culture completed 12.09 p. m.

Serum test.		Culture checks.	
Serum used.	Hours of survival.	Cubic centimeters of culture used—2 mice to each dose.	Hours of survival.
Control serum P_1C	72 S S	0.00,000,1	29 43
Test serum X	(11) (41) S	0.00,000,01	39 41
Test serum Y	S S S	0.00,000,001	42 43
Test serum Z	29 S S	0.00,000,000,1	49 S
Control serum P_1C	(90) 37 S		

Date: Oct 17, 1919.
Operator: A. B. C.